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# Controlled Delivery of Diphtheria Toxoid Using Biodegradable Poly(D,L-Lactide) Microcapsules

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Diphtheria toxoid, which is an important vaccine in the expanded program of immunization (EPI) in the developing countries, was microencapsulated using poly(D,L,-lactide) of 49,000 molecular weight and the in-water drying technique. The microcapsules were subjected to an *in vitro* antigen release study using a sensitive enzyme-linked immunosorbent assay (ELISA) developed in the laboratory. Antibody titers in immunized Balb/C mice were also determined using direct ELISA. The antibody units in the immunized group till day 75 were quite comparable to those in the group receiving conventional three-dose injection of diphtheria toxoid with calcium phosphate as an adjuvant. SEM photographs of the microcapsules during *in vitro* degradation demonstrated the erosion kinetics of the polymer, leading to controlled release of the antigen.

KEY WORDS: vaccine delivery; biodegradable microcapsules; antigen ELISA; erosion kinetics; antibody titers.

## INTRODUCTION

Controlled-release technology has recently shifted its emphasis from low molecular weight drugs to high molecular weight macromolecules, because many of the future drugs will be of recombinant DNA origin having high molecular weights. This development has led to newer polymers with a greater degree of biocompatibility and reproducible degradation kinetics (1,2). Vaccines are an example requiring novel controlled-release technology (3–5). Most vaccines require two or three primary immunizations, followed by a booster for optimum immune response. If one injection of the immunization schedule is missed, it leads to manifold loss of effective antibody titers. According to WHO statistics, more than 30% of the patients do not return for the next injection at each time point of the immunization schedule. The impact of noncompliance is most severe in the third world countries, where more than a million children die each year from vaccine-preventable diseases.

Ideally one would like to see the development of a controlled-delivery system that would release two or three doses of the vaccine in a programmable manner at one "single contact point administration." Such one-time vaccination under an expanded program of immunization would reach a greater percentage of the target population and afford protective antibody titers.

Diphtheria toxoid (MW 62,000) was choosen as a model vaccine, as it is a common vaccine in the immunization schedule worldwide and the toxoid, being a denatured protein, presents few stability problems. Also, the immunogenicity of the toxoid is well recorded. Stability studies carried out at this institute on β-hcG-DT, an antifertility vaccine, has shown that the carrier DT is stable at 37°C for 12 months, with no loss of immunogenicity either *in vitro* or *in vivo*.

In the present study, diphtheria toxoid (DT) was microencapsulated using biodegradable poly(D,L-lactide) polymer, and the *in vitro* release monitored using an enzyme-linked immunosorbent assay (ELISA). The immune response to the antigen was determined in Balb/C mice. The antibody titers, between a group receiving a conventional dose of DT with calcium phosphate as an adjuvant and a group receiving subdermally implanted microspheres, were compared.

## MATERIALS AND METHODS

### Materials

Diphtheria toxoid (MW 62,000) having a concentration of 3500 Lf/ml (limes flocculation, the International Unit for vaccines) and a protein concentration of 15 mg/ml, was obtained from Serum Institute of India, Pune. Poly(D,L-Lactide) was obtained from Birmingham Polymers Inc. (Birmingham, AL) and Boehringer Ingelheim (FRG). Dichloromethane, polyvinylpyrrolidone and polystyrene standards were obtained from Aldrich Chemical Company, Inc. (Madison, WI). D,L-Lactic acid was obtained from Sigma Chemical Company (St. Louis, MO). The other chemicals were obtained from commercial suppliers and were used as received.

### Quantitative Estimation of Diphtheria Toxoid

Diphtheria toxoid (DT) was measured by an enzyme-linked immunosorbent assay (ELISA) to estimate *in vitro* release rates from microcapsules and *in vivo* antibody titers in mice (6–9). Antigen (DT) detection was carried out using polyclonal sera containing anti-DT antibodies raised in goats to estimate the amount of antigen being released in the dispersion medium by the microcapsules. The 96-well ELISA plate was coated with increasing concentrations of DT in 50 m*M* coating phosphate buffer of pH 7.4 (from 10 to 100 ng/well). The plate was incubated at 37°C for 1 hr and then washed with phosphate buffer saline (50 m*M*, pH 7.4) with 0.2% Tween 20 (washing buffer). Then 100 μl of the antiserum (diluted 1:800) was added to each well, and the plate again incubated at 37°C for 1 hr. After incubation the plate was again washed with washing buffer thrice at an interval of 5 min between each washing. One hundred microliters of the conjugate Prot*A horse radish peroxidase (dilution, 1:25,000) was added to each well and the plate kept for incubation at 37°C for 1 hr. After incubation the plate was washed with the washing buffer thrice, and 100 μl of the substrate (0.05% of *O*-phenylenediamine and 0.1% of hydrogen peroxide in citrate phosphate buffer) was added to each well. The plate was incubated at 37°C for 15 min and then the reaction in each well was stopped with the addition of 50 μl 5 *N* sulfuric acid. Absorbance was read at 492 nm on an ELISA plate reader (Eurogenetics, NV, Belgium). The ab-

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sorbance-vs-concentration plot was linear from 10 to 100 ng. Each unknown sample was run with a standard curve in duplicate.

For the determination of the antibody units *in vivo* the assay principle was the same, the only difference being the coating of fixed antigen concentration initially and adding varying dilutions of the standard and test antisera. The second antibody was not limiting in these estimations. The antibody units were calculated by multiplying the dilution of the test sample by its absorbance reading.

### Polymer Synthesis

For the above study both presynthesized commercial polymers and polymers synthesized in our laboratory were used. Poly(D,L-lactide) was synthesized using 160 g D,L-lactic acid monomers and 6 g activated ion-exchange resin (Dowex). The polycondensation reaction was performed at 185°C for 8 hr under vacuum and constant stirring. The resultant polymer had a low molecular weight (6000) as determined by gel permeation chromatography (GPC). A Waters GPC system was used with Ultrastrygel coulmns and a refractive index detector. The eluent used was tetrahydrofuran (THF) at 30°C and a flow rate of 1.0 ml/min. The commercial presynthesized polymers were also subjected to molecular weight determination, in comparison with standard polystyrene samples in THF. Poly(D,L-lactide) obtained from Birmingham Polymers, having a viscosity of 0.75 dl/g and a molecular weight of 49,000, was also used because the low molecular weight of the synthesized polymers made them unsuitable for long-term release study.

### Microencapsulation

The vaccine was microencapsulated using the in-water drying method (10–13). To 1 ml of PBS (50 m*M*, pH 7.4), 150 Lf units of the vaccine and 100 mg of gelatin were added. Poly(D,L,-lactide) (1 g) was dissolved in 10 ml dicholoromethane. This organic solution was gradually added to the aqueous phase containing the vaccine with high-speed mixing on a ultrasonicator to form a fine emulsion. The temperature was lowered to 10°C by keeping the emulsion in ice to increase its viscosity. This viscous emulsion was not added drop by drop to a 0.1% polyvinylpyrrolidone solution in water with stirring to yield a w/o/w emulsion. The minute globules separated to form distinct microcapsules. The microcapsules were agitated for 2 hr to aid complete solvent evaporation. Finally, the microcapsules were filtered and vacuum-dried.

To determine actual vaccine loading, 10 mg of the microcapsules was crushed and dispersed in 1 ml of PBS, and DT was determined by ELISA. To each of 10 vials containing 1 ml of PBS, 10 mg of the microcapsules was added. These vials were placed at 37°C for *in vitro* release rate studies. One vial was estimated for its DT content in the dispersion medium each week by ELISA.

For the *in vitro* studies, three groups of 10 mice each were taken (3-month-old inbred strain of Balb/C mice). To one group, microcapsules equivalent to 3 Lf units were subcutaneously implanted inside the right thigh. To the second group, three injections of 1 Lf of DT with calcium phos-

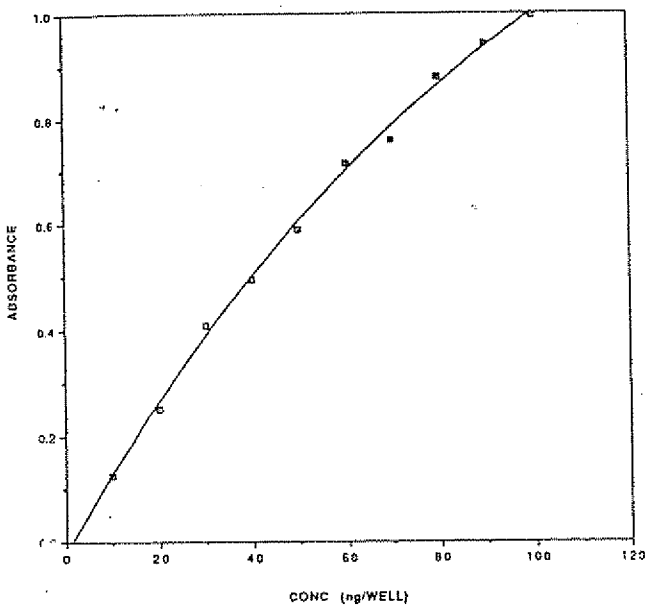


Fig. 1. Quantitative estimation of DT antigen using the developed ELISA at 492 nm. The sensitivity is 2 ng and the linearity ranges from 10 to 100 ng.

phate, each at an interval of 30 days, were given, i.e., days 0, 30, and 60. The third group served as a control.

The initial microcapsules and those retrieved after 21 days of *in vitro* degradation were subjected to scanning electron microscopic studies to determine the surface uniformity and the release characteristics. A 35 JEOL SEM instrument with 100-Å gold–palladium coating was used for this study.

## RESULTS AND DISCUSSION

Poly(D,L-lactide) of 49,000 molecular weight was selected for long-term release rate studies of the toxoid. The in-water drying method gave microcapsules in the range of 30–100 μm. As this range of microcapsules can pass through

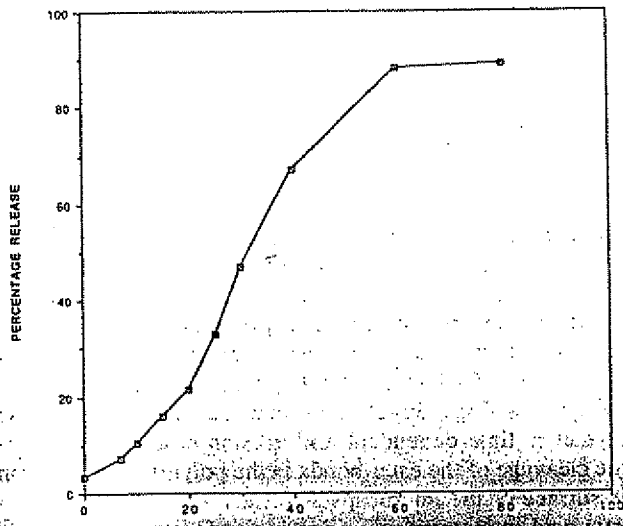


Fig. 2. *In vitro* release of the antigen from poly(D,L-lactide) microcapsules calculated as the percentage of the amount released at time to the actual vaccine loading.

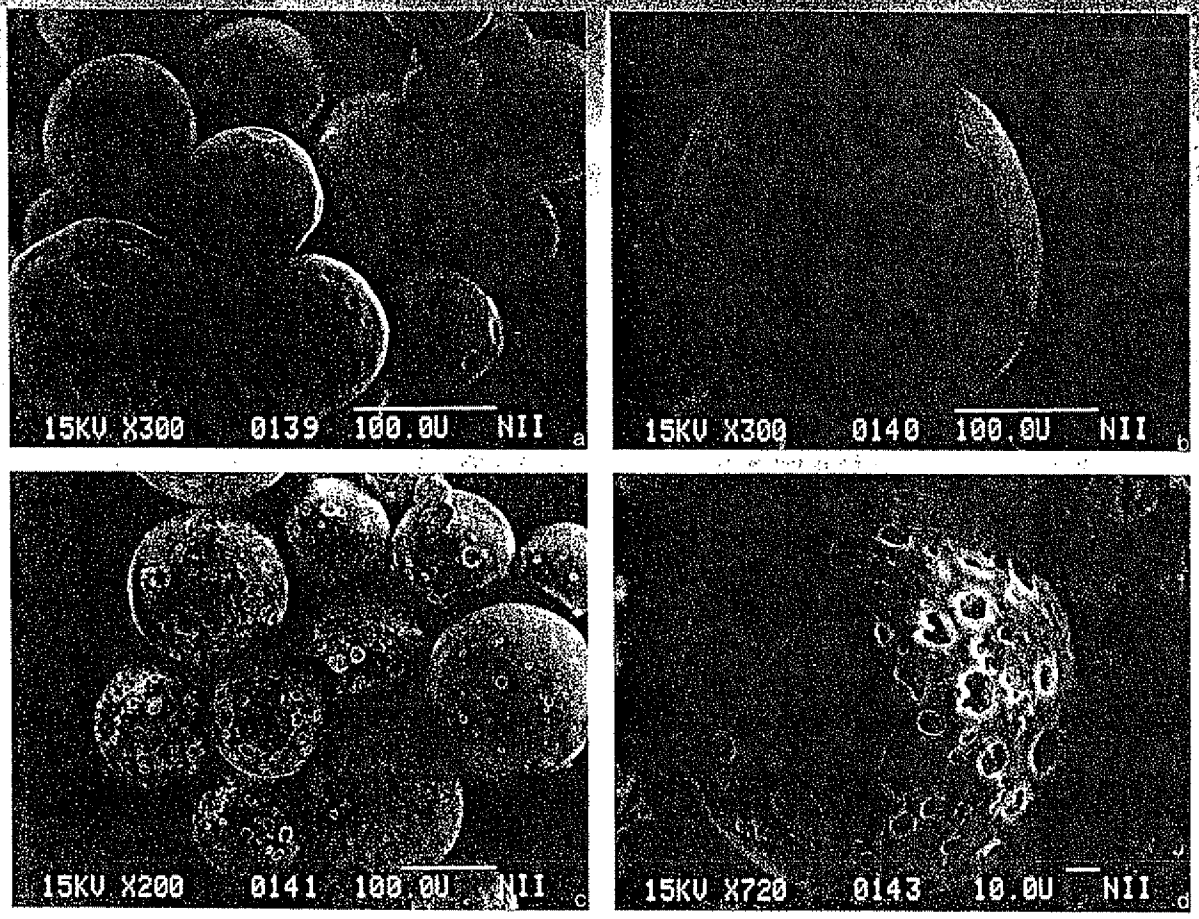


Fig. 3. SEM of poly(D,L-lactide)/DT microcapsules after complete solvent evaporation and vacuum drying. (b) SEM of a large microcapsule to exhibit surface uniformity and lack of surface porosity before initiating *in vitro* degradation. (c) SEM of poly(D,L-lactide)/DT microcapsules after 21 days of *in vitro* degradation in 50 m*M* PBS at pH 7.4. (d) SEM of poly(D,L-lactide)/DT microcapsule after 21 days of *in vitro* degradation exhibiting surface geometry and high porosity leading to greater water uptake. (a, b) ×300, (c) ×200, and (d) ×720; reduced 35% for reproduction.

an 18-gauge hypodermic needle, the preparations were not separated on basis of their size. The actual Lf units of DT in the microcapsules was determined to be 93% of theoretical. The ELISA for the detection of *in vitro* release rates of DT was highly sensitive within the range of 10–100 ng (Fig. 1) and all samples were diluted to fall within this sensitivity range.

The *in vitro* DT release from the microcapsules was 88% of the actual vaccine loading in 60 days (Fig. 2). Thus the microcapsule matrix erodes sufficiently over 60 days to allow depletion of the macromolecule through development of pores and craters on its surface, seen in the SEM studies. During the process of microencapsulation, a small loss of antigenic determinants could have occurred as the ELISA detected only 88% of the total vaccine loading. The SEM photographs (Figs. 3a–d) show that the degradation of the polymer is time dependent and erosion based. The hydrolytic cleavage of the ester bonds in the polymer backbone on the surface of the microcapsules leads to its degradation to lactic acid monomers, resulting in the formation of craters and channels through which the antigen is released. As the erosion is time dependent, a gradual but continuous release of the antigen occurs from the microcapsule.

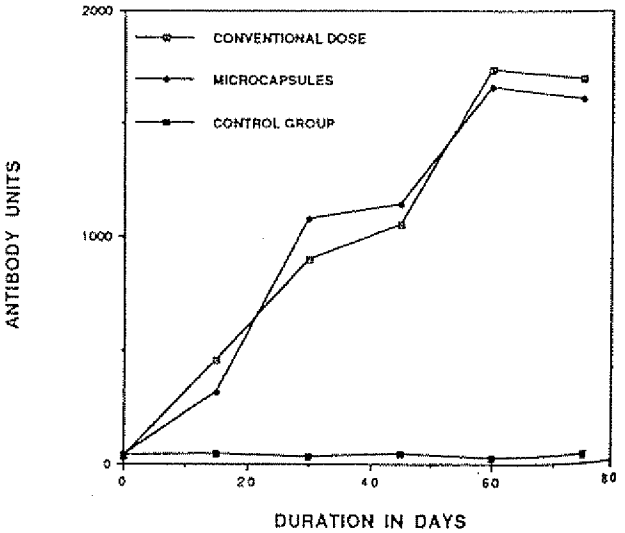


Fig. 4. *In vivo* antibody titers in the three groups of BALB/c mice. The antibody units correspond to the product of the dilution of the serum to its absorbance. (—⊡—) Group receiving conventional three-injection schedule; (—♦—) group receiving poly(D,L-lactide) microcapsules; (—■—) the control group.

To predict the vaccine release from theoretical considerations, the following equation can be applied, which is suggested for an erosion-based release of the drug (14).

$$M_t / M_\infty = 1 - (1 - K_0 t / C_0 a)^n$$

where $M_t/M_\infty$ = fractional drug release
$K_0$ = device erosion constant
$C_0$ = initial drug concentration uniformly distributed
$a$ = radius of sphere or half-thickness of the slab
$n$ = shape term: 1 for a slab, 2 for a cylinder, and 3 for a sphere
$t$ = time period

Therefore, by determining the degradation rate of a polymer of known molecular weight and knowing its initial drug concentration, it is possible to predict the release rate of the drug molecule occurring only through erosion. Another important parameter controlling the rate of degradation of the polymer is the pH and ionic strength of the dispersion medium (15–17). Further, as the degradation of the polymer progresses, it becomes more hydrophilic and shows greater water intake because of the surface geometry of the degrading polymer, leading to accelerated degradation and depletion of the matrix (18).

The antibody titers till day 75 in the group immunized with the microcapsules were comparable with those in the group receiving the conventional three-injection schedule (Fig. 4). Therefore a slow antigen release yields comparable antibody titers and did not seem to develop any tolerance in the animal model. Whether a triphasic pulsatile release would offer a better result remains to be studied.

We conclude that a denatured protein preparation such as DT can be encapsulated using poly(D,L-lactide) without loss of its immunogenicity. The continuous release of the antigen elicits antibody titers over 75 days that were comparable to those obtained with the conventional toxoid dose with calcium phosphate as an adjuvant.

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